Immunity
Review

Development and Maintenance of Regulatory T cells

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Regulatory T (Treg) cells are a developmentally and functionally distinct T cell subpopulation that is engaged in sustaining immunological self-tolerance and homeostasis. The transcription factor Foxp3 plays a key role in Treg cell development and function. However, expression of Foxp3 alone is not sufficient for conferring and maintaining Treg cell function and phenotype. Complementing the insufficiency, Treg-cell-specific epigenetic changes are also critical in the process of Treg cell specification, in regulating its potential plasticity, and hence in establishing a stable lineage. Understanding how epigenetic alterations and Foxp3 expression coordinately control Treg-cell-specific gene regulation will enable better control of immune responses by targeting the generation and maintenance of Treg cells.

Introduction

Naturally occurring $CD25^+CD4^+$ regulatory T (Treg) cells, which constitutively express the transcription factor Foxp3, are indispensable for the maintenance of immune self-tolerance and homeostasis by suppressing aberrant or excessive immune responses harmful to the host (Rudensky, 2011; Sakaguchi et al., 2008). The majority of $Foxp3^+$ natural Treg (nTreg) cells are produced by the thymus as an antigen-primed and functionally mature T cell subpopulation specialized for immune suppression. Some of them also differentiate from naïve conventional T (Tconv) cells in the periphery under certain conditions. The main task of $Foxp3^+$ nTreg cells is to migrate to inflammation sites and suppress various effector lymphocytes, especially helper T (Th) cell subsets: Th1, Th2, Th17, and follicular Th (Tfh) cells (Chaudhry et al., 2009; Chung et al., 2011; Koch et al., 2009; Linterman et al., 2011). These features of nTreg cells raise the issues of how their cell fate as a distinct cell lineage is determined in the thymus; how their functional and phenotypic stability is maintained in the periphery with certain adaptability to inflammatory environments; and how peripherally induced Treg (iTreg) cells are different from thymus-produced ones in the mode of their generation, cell fate, and functional stability. These issues are also relevant in clinical settings to enrich and expand functionally stable Treg cells for immune suppression or to specifically deplete Treg cells or attenuate their suppressive activity for enhancing immune responses.

There is substantial evidence that Foxp3, a forkhead transcription factor encoded by the X chromosome, plays a critical role in the development and function of Treg cells (Fontenot et al., 2003; Hori et al., 2003). Mutations of human *FOXP3* result in impaired development or dysfunction of Treg cells and, consequently, the occurrence of immunodysregulation polyendocrinopathy enteropathy X-linked syndrome accompanying severe autoimmune diseases, inflammatory bowel disease, and allergy (Bennett et al., 2001). Likewise, mice that carry a mutation or genetic deletion of *Foxp3* are deficient in Treg cells and develop fatal systemic autoimmunity (Brunkow et al., 2001; Fontenot et al., 2003). In addition, forced expression of Foxp3 is able to confer Treg-cell-like suppressive activity on Tconv cells (Fontenot et al., 2003; Hori et al., 2003). Foxp3 has therefore been considered as a lineage-specifying transcription factor of Treg cells or a master regulator of its functions.

There are, however, several indications that Foxp3 expression per se might not be sufficient for stably maintaining Treg cell suppressive function or reliably delineating functional Treg cells. For example, activated human Tconv cells transiently express FOXP3 at a low level without acquiring Treg cell suppressive activity (Allan et al., 2007). $CD4^+$ T cells in human peripheral blood contain a $FOXP3^+$ T cell subpopulation that does not exhibit Treg cell suppressive activity and even produces proinflammatory cytokines upon activation (Miyara et al., 2009). Analysis of $Foxp3^{gfp}$ mice, in which *Foxp3* is disrupted by the insertion of the gene encoding GFP, demonstrated that $Foxp3^-GFP^+$ T cells express some Treg cell signature genes, such as *Ctla4* and *Il2ra* (Gavin et al., 2007; Lin et al., 2007). Thus, not all of the $Foxp3^+$ T cells are functional Treg cells, and Treg cell signature molecules can be expressed, at least to a certain extent, without Foxp3. Consistently, ectopic retroviral transduction of Foxp3 in Tconv cells has failed to induce the expression of many Treg cell signature genes (Hill et al., 2007; Sugimoto et al., 2006). In addition, Treg cell clones or lines maintained in vitro are functionally stable (Levings et al., 2001). Also, an in vivo cell-fate-chasing study has shown that $Foxp3^+$ nTreg cells represent a stable cell lineage that is capable of sustaining Foxp3 expression and suppressive function over multiple rounds of cell division, even in inflammatory environments (Rubtsov et al., 2010). Taken together, these findings suggest that Treg cell development is not quite as simple as the widely accepted Foxp3-centered scheme that Foxp3 expression is sufficient to define nTreg cells or to determine their cell fate and function. An additional molecular event (or events) might complement Foxp3 function in the generation of Treg cells and the maintenance of their function and phenotype.

In this review, we discuss the molecular basis of Treg cell development and function by focusing on how epigenetic regulation and Foxp3 expression independently and coordinately control Treg cell development and function.

Epigenetic Changes in Treg Cells

Epigenetic modifications, which include histone modifications, DNA methylation, microRNAs, nucleosome positioning, chromatin interaction, and chromosome conformational changes, play indispensable roles in cell differentiation, especially for cell-lineage stabilization (Gibney and Nolan, 2010; Kim et al., 2009; Musri and Párrizas, 2012). In particular, DNA methylation and histone modifications critically contribute to cell-lineage determination and maintenance because they are heritable through cell divisions. Genomic DNA is mainly methylated by DNA methyltransferases (Dnmt family members), whereas it can be demethylated by multiple steps, including methylcytosine hydroxylation mediated by TET family members (Bhutani et al., 2011; Pastor et al., 2011). Similarly, histones are modified for gene activation or repression by acetylation or deacetylation, methylation or demethylation, and phosphorylation or dephosphorylation (Teperino et al., 2010). Thus, epigenetic status is reversible. It is also known, however, that DNA methylation status modified in the early stages of development, such as genomic imprinting, is stably maintained throughout subsequent differentiation processes. Epigenetic changes of some specific loci are also stably sustained in specific cell lineages, including Treg cells (Ansel et al., 2003; Ohkura et al., 2012; Schmidl et al., 2009; Thomas et al., 2012).

Recent genome-wide analyses have revealed several regions that show different patterns of DNA methylation or histone modification between Tconv and Treg cells in humans and mice (Floess et al., 2007; Ohkura et al., 2012; Schmidl et al., 2009; Wei et al., 2009). For example, Treg-cell-specific DNA hypomethylation occurs in a limited number of loci, half of which are located in small regions within gene bodies (exons and introns). Such genes with Treg-cell-specific DNA hypomethylation include those encoding Treg-cell-function-associated or Treg-cell-specific molecules, such as Foxp3, CTLA-4, and Eos (Ohkura et al., 2012). Many of the differentially methylated regions harbor DNA-methylation-dependent enhancer activity in reporter-gene assays (Schmidl et al., 2009). Furthermore, some of the Treg-cell-specific changes in DNA methylation are highly stable in Treg cells, whereas others are not. For example, *Foxp3* intron 1 (corresponding to *Foxp3* conserved noncoding sequence 2 [CNS2]), *Ctla4* exon 2, and *Ikzf4* (encoding Eos) intron 1, are specifically demethylated in nTreg cells, and the hypomethylation is stable after T cell receptor (TCR) stimulation, cell proliferation, or cytokine treatments (e.g., with IL-2 or TGF-β) (Ohkura et al., 2012). In contrast, the DNA methylation status of *Il2ra* intron 1, which is demethylated in nonactivated Treg cells, is relatively unstable and becomes demethylated in Tconv cells by mere in vitro culture with or without TCR stimulation. In addition, enhanced H3K4me3 histone modification of the Treg cell signature genes detected in nTreg cells is easily primed in Tconv cells under a Th1-, Th2-, or Th17-cell-polarizing or iTreg-cell-inducing condition (Wei et al., 2009) (Figure 1). In line with these findings, a high-resolution DNaseI footprint analysis has shown that specific alterations in chromatin accessibility occur in Treg cells in the course of their differentiation from their precursors (Samstein et al., 2012). Although the DNaseI-hypersensitive regions do not differ mostly between $CD4^+Foxp3^-$ T cells and $CD4^+Foxp3^+$ T cells, a small number of genes show increased hypersensitivity in Treg cells, indicating specific alterations in local nucleosome positioning and chromatin accessibility. The loci identified as newly accessible in Treg cells are enriched in the genes known to be critical for Treg cell function, such as *Foxp3*, *Ctla4* and *Ikzf2*. They are also classified as genes possessing Treg-cell-specific DNA hypomethylated regions in Treg cells, as discussed above (Ohkura et al., 2012; Schmidl et al., 2009). Thus, Treg cells acquire and sustain highly specific and stable epigenetic changes as exemplified by DNA hypomethylation at specific loci of a limited number of genes. This Treg-cell-specific DNA hypomethylation is a reliable marker for assessing the epigenetic status of Treg cells.

The Molecular Basis of Treg-Cell-Specific DNA Hypomethylation

DNA hypomethylation is generally associated with an open chromatin structure, and one of the molecular consequences is increased accessibility of transcription factors to particular genomic regions for the enhancement of transcription. A luciferase reporter assay incorporating the methylated or demethylated *Foxp3* CNS2 region has indeed shown that its demethylation substantially increases the gene transcription in Treg and non-Treg cells, indicating that demethylation promotes gene expression by utilizing molecules commonly present in Treg and non-Treg cells (Polansky et al., 2010) (Figure 2). Methylated DNA is known to be bound by factors such as DNA-methyl-binding-domain proteins, methyl CpG-binding proteins, Kaiso, and some ZBTB proteins, and these factors assist the repression of target-gene transcription by preventing the binding of transcription factors and recruiting HDAC and histone methyltransferases (Tost, 2010). Although the order of molecular events involving histone modification, DNA methylation, and chromatin remodeling is a subject of much debate, demethylation of DNA is suggested to interfere with the action of these transcriptional repressors and instead allow the binding of other factors. In fact, Ets-1 specifically binds to the demethylated CNS2 region of *Foxp3* (Polansky et al., 2010). This region is also bound by Foxp3, Gata-3, and STAT5 in Treg cells, either directly or indirectly, and targeted deletion of the *Foxp3* CNS2 region or *Gata3* in Treg cells results in reduced transcription activity of *Foxp3* (Rudra et al., 2012; Yao et al., 2007; Zheng et al., 2010). Collectively, DNA demethylation of the CNS2 region allows enhanced gene expression at the *Foxp3* locus. It needs to be examined whether other gene loci specifically demethylated in Treg cells exhibit similar enhancing effects.

Roles of Epigenetic Changes and Foxp3 Expression in Treg Cells

With the presence of the Treg-cell-specific DNA hypomethylation pattern in nTreg cells, it can be asked when it is installed in Treg cells, how it contributes to Treg-cell-type gene expression, and how its contribution is distinct from that of Foxp3. In the thymus, the Treg-cell-specific epigenetic pattern becomes evident in developing Treg cells or their precursors at the CD4-single-positive stage; it progresses from the thymus to the periphery to become firmly established (Ohkura et al., 2012). Importantly, in *Foxp3*-null mice that express GFP from the *Foxp3* promoter but fail to express Foxp3, $GFP^+CD4^+CD8^-$ thymocytes acquire the Treg-cell-type DNA hypomethylation pattern to an extent and with a time course similar to those of

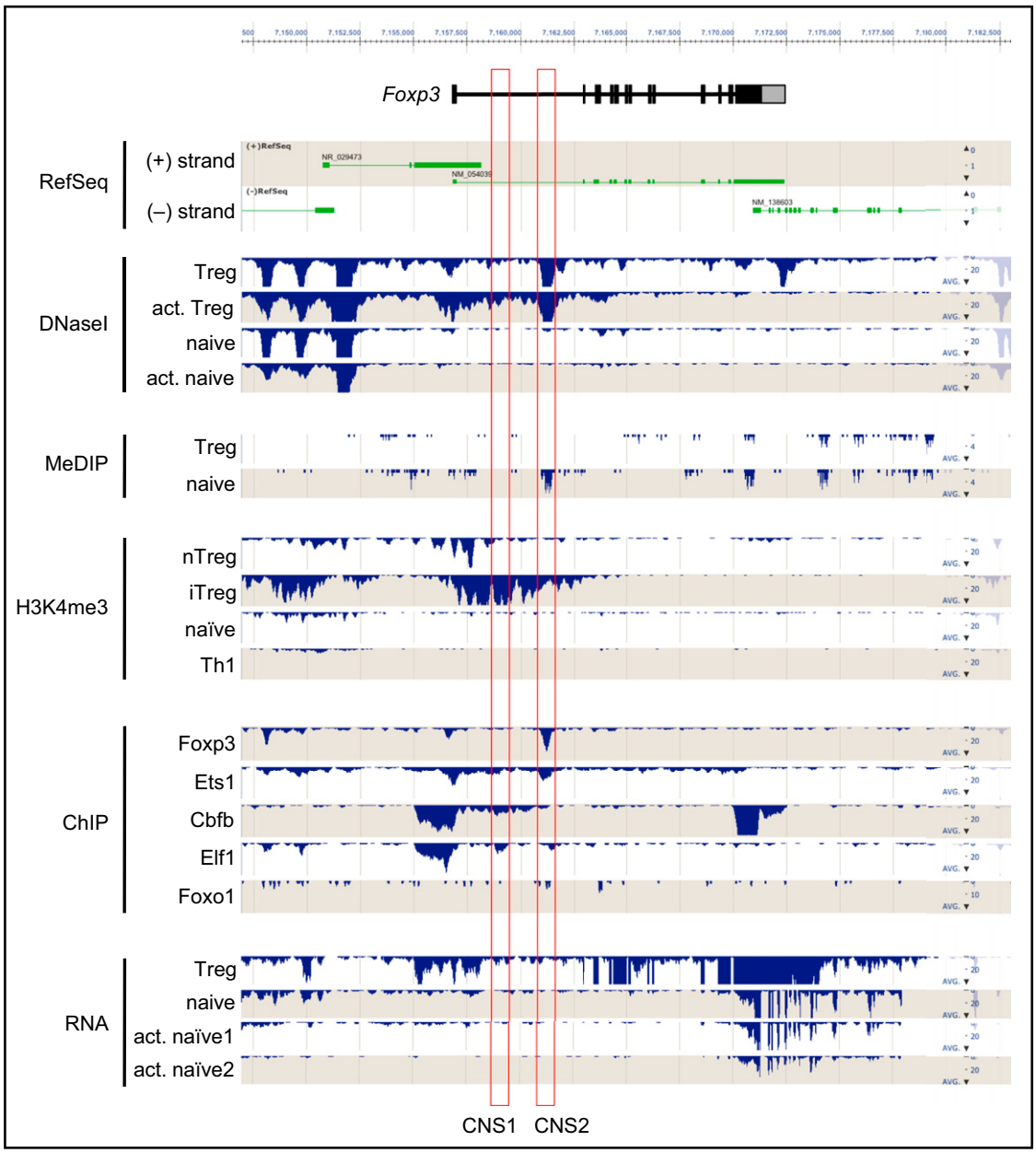

Figure 1. Epigenetic Status of *Foxp3*

A schematic representation of *Foxp3* shows the reference sequence (RefSeq) of the gene locus, DNaseI-hypersensitive regions determined by DNaseI sequencing (DNaseI) (Samstein et al., 2012), methylated DNA regions by methylated DNA immunoprecipitation sequencing (MeDIP) (Ohkura et al., 2012), H3K4me3 modification by chromatin immunoprecipitation (ChIP) sequencing (H3K4me3) (Wei et al., 2009), ChIP sequencing against transcription factors (Samstein et al., 2012; Zheng et al., 2007), and RNA expression by RNA sequencing (RNA) (unpublished data). Red rectangles indicate *Foxp3* CNS1 and CNS2 regions. "Act. naïve1" and "act. naïve2" represent naïve T cells stimulated with CD3 and CD28 antibodies (naïve1) and with PMA and ionomycin (naïve2).

normal Foxp3-expressing thymocytes (Ohkura et al., 2012). The whole-gene expression pattern is also similar between nTreg cells and those installed with the Treg-cell-specific epigenetic pattern but not expressing Foxp3. These findings collectively indicate that Treg-cell-type DNA hypomethylation is installed in developing Treg cells without Foxp3 expression and contribute to the whole-gene expression profiles in steady-state Treg cells.

Treg-cell-specific DNA hypomethylation and Foxp3 expression have distinct roles in establishing Treg-cell-type gene expression. Foxp3 represses expression of some key molecules for Treg cell function, such as IL-2, IFN-γ and Zap70, whereas Treg-cell-specific epigenetic changes do not (Ohkura et al., 2012). Treg-cell-specific DNA-hypomethylation-dependent gene regulations, e.g., enhancement of *Ikzf2* and *Ikzf4* expression, are independent of Foxp3. Supporting this possible division of labor, Treg-cell-specific DNA hypomethylated regions in the genome are mostly different from Foxp3-binding sites (Ohkura et al., 2012; Samstein et al., 2012; Zheng et al., 2007). Moreover,

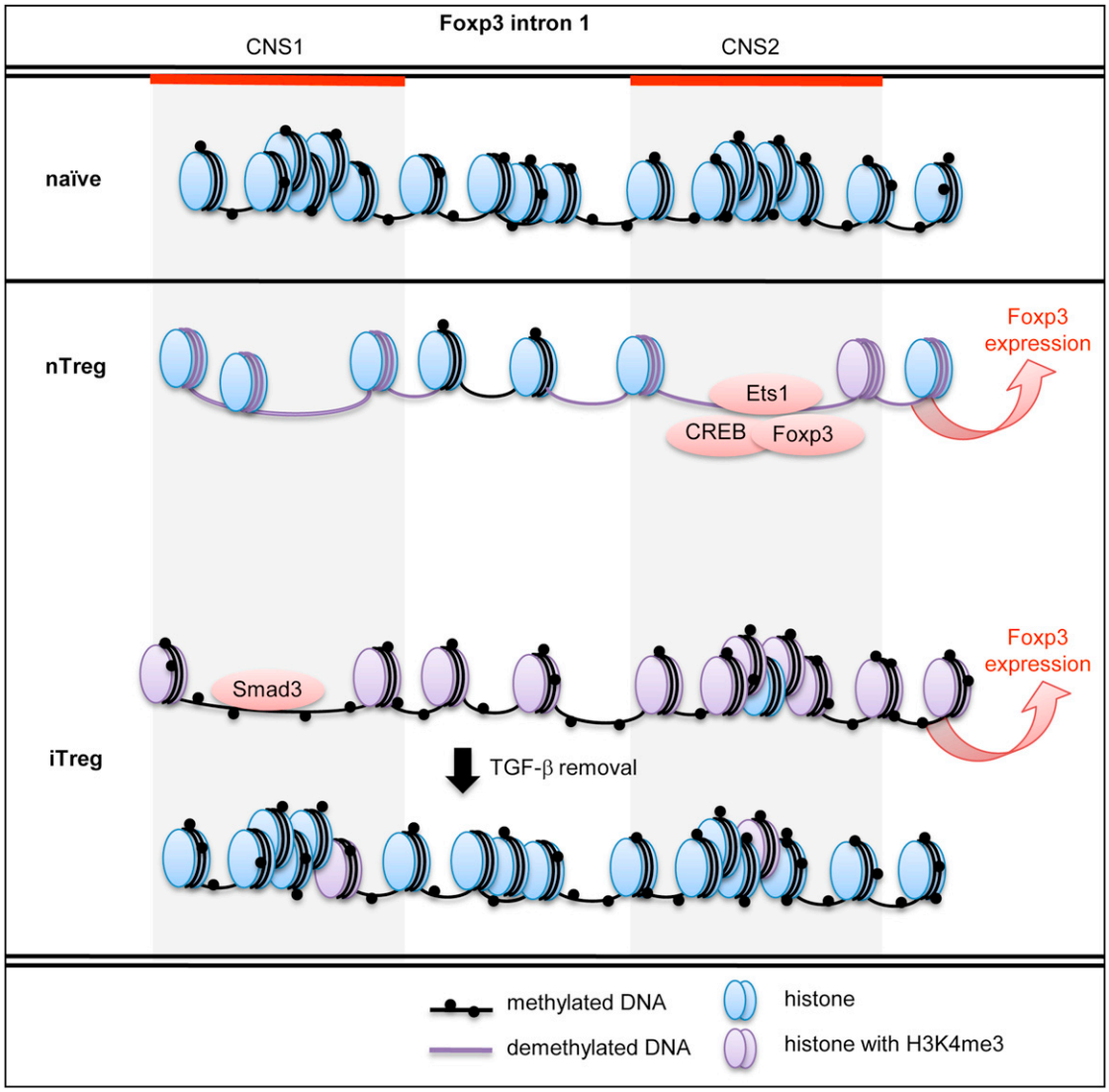

Figure 2. Functional Significance of Treg-Cell-Type Epigenetic Changes
In nTreg cells, specific DNA hypomethylation was observed in the *Foxp3* CNS2 region, in which several transcription factors (including Ets1, CREB, and Foxp3 itself) assemble and activate Foxp3 expression, irrespective of the extracellular stimulations, such as TGF-β. In contrast, for in vitro iTreg cells induced by TGF-β, DNA hypomethylation does not occur in either the CNS1 or the CNS2 region, whereas H3K4me3 modification is enhanced in the CNS1 region, in which Smad3 locates and activates Foxp3 expression. After removal of TGF-β, Foxp3 expression is not maintained in these iTreg cells.

chromatin accessibility of the Foxp3-binding sites is similar between Treg and Tconv cells (Samstein et al., 2012), suggesting that Foxp3-dependent gene regulation is independent of Treg-cell-specific epigenetic changes. Notably, however, both Foxp3 expression and Treg-cell-type DNA hypomethylation are involved in controlling the expression of some Treg-cell-function-associated molecules, such as *Il2ra*, *Ctla4*, and *Foxp3* itself, especially upon TCR stimulation (Hill et al., 2007; Hori et al., 2003; Ono et al., 2007). Taken together, it is likely that Foxp3-dependent gene regulation and Treg-cell-epigenome-dependent regulation have distinct roles in determining the whole Treg-cell-type gene expression pattern and complement mutually in the expression of certain specific genes in Treg cells.

What, then, induces Foxp3 and the Treg cell epigenome in developing Treg cells in the thymus and the periphery? TCR stimulation is an important factor for both events (Jordan et al., 2001; Kawahata et al., 2002; Ohkura et al., 2012), yet it seems that the quality of TCR stimulation required for Foxp3 induction or Treg-cell-epigenome installation is different. Foxp3 expression in developing thymocytes appears to depend chiefly on the intensity of TCR stimulation (Jordan et al., 2001; Kawahata et al., 2002) and is rapidly induced after TCR stimulation (Figure 3). In contrast, the acquisition of Treg-cell-specific DNA hypomethylation appears to depend on the duration of TCR stimulation (Ohkura et al., 2012). It is thus likely in thymic Treg cell development that only those thymocytes that have acquired Treg-cell-type epigenetic changes with TCR stimulation become poised for expressing Foxp3 and consequently express Foxp3 by receiving an appropriate strength of TCR stimulation and thereby develop into stable Foxp3$^+$ Treg cells. This indicates

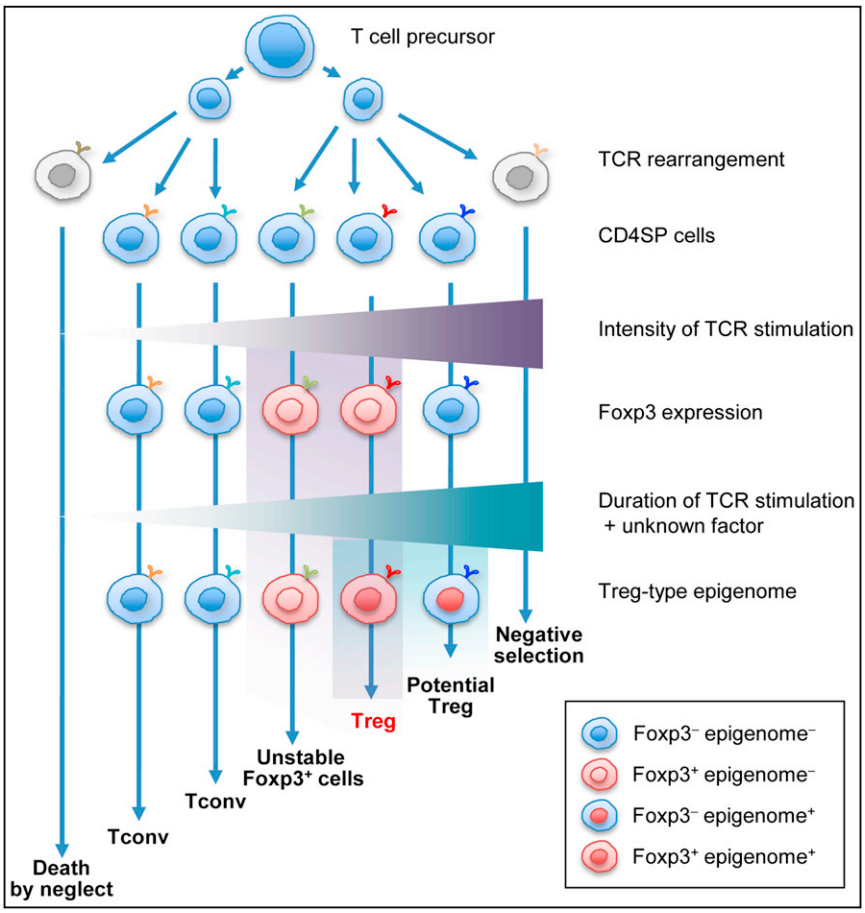

Figure 3. A Model for Treg Cell Development in the Thymus

In developing T cells in the thymus, TCR gene rearrangement generates diverse TCRs that recognize self-ligands at various intensities and durations (shown as gradients). TCR stimulation with relatively higher intensities (but below the threshold required to induce apoptosis) induces Foxp3 expression, whereas TCR stimulation for an appropriate length of time produces the Treg-cell-type DNA hypomethylation pattern. Developing T cells that happen to have both events (Foxp3$^+$ epigenome$^+$ T cells) are driven to a stable Treg cell lineage. Foxp3$^+$ T cells without the accompanying Treg-cell-type epigenome (Foxp3$^+$ epigenome$^-$) are unstable and might lose Foxp3 expression, whereas T cells with the Treg-cell-type epigenome but without Foxp3 expression (Foxp3$^-$ epigenome$^+$) are ready to express Foxp3 and are capable of differentiating into functional Treg cells. T cells that recognize self-ligands too strongly are negatively selected by apoptosis, whereas those that recognize self-ligands too weakly fail in positive selection (death by neglect).

that those thymocytes having acquired the Treg-cell-type epigenetic changes but not expressing Foxp3 would easily differentiate into functional Treg cells, whereas Foxp3-expressing thymocytes without the epigenetic pattern would lose Foxp3 expression and fail to differentiate into Treg cells. Thus, Treg cell development appears to be multistep processes (with a stochastic element) involving Foxp3 induction and the establishment of a Treg-cell-specific epigenetic pattern.

Differences between Thymic Treg and iTreg Cells

Although thymus-derived nTreg cells, in-vitro-generated iTreg cells, and in-vivo-induced iTreg cells bear similar phenotypes, such as expression of Foxp3, CD25, and CTLA-4, their epigenetic status is different. There is a substantial amount of data indicating that DNA methylation status, especially at the *Foxp3* CNS2 locus, is similar between thymic Treg cells and in-vivo-induced iTreg cells but different between in-vivo-induced iTreg cells and in-vitro-induced cells (Ohkura et al., 2012; Polansky et al., 2008). Because DNA demethylation of the *Foxp3* CNS2 locus mainly contributes to the stability of Foxp3 expression, nTreg cells are able to stably maintain Foxp3 expression irrespective of extracellular conditions, Foxp3-inducing signals, or cell proliferation. In contrast, Foxp3$^+$ iTreg cells induced in vitro by TGF-β and/or retinoic acids are less stable in the expression of Treg-cell-function-associated molecules, such as Foxp3 and CTLA-4 (Hill et al., 2007; Ohkura et al., 2012; Polansky et al., 2010). In vitro TGF-β treatment can change histone modification status of the *Foxp3* CNS1 region, where several transcription factors assemble and transactivate Foxp3 expression, but fail to induce Treg-cell-type DNA hypomethylation in the *Foxp3* CNS2 region (Ohkura et al., 2012; Samstein et al., 2012; Wei et al., 2009) (Figure 2). In addition, TGF-β-induced Foxp3 expression is lost after removal of TGF-β or TCR stimulation in vitro (Floess et al., 2007; Selvaraj and Geiger, 2007), suggesting instability of the expression. A recent report has also shown that thymus-derived nTreg cells and mucosa-generated iTreg cells can be distinguished by the expression levels of neuropillin 1 (Nrp1) (Weiss et al., 2012). Similar to Foxp3$^+$Nrp1$^+$ thymic Treg cells, Foxp3$^+$Nrp1$^-$ iTreg cells, which retain DNA demethylation of the *Foxp3* CNS2 region to some extent, are stable and suppress immune responses in vitro and in vivo. These findings confirm the importance of Treg-cell-specific DNA demethylation in cell stability of in-vivo-generated iTreg cells.

In the case of some in-vivo-induced iTreg cells, particularly those generated in gut-associated lymphoid tissues, Foxp3 expression depends on cytokine milieu, which might lead to H3K4me3 modification of the CNS1 region. These in-vivo-induced Foxp3$^+$ T cells gradually acquire DNA demethylation in the *Foxp3* CNS2 region by TCR stimulation (Ohkura et al., 2012). The differences in developmental processes between thymus-derived nTreg cells and in vivo iTreg cells are illustrated by the germ-line deletion of the *Foxp3* CNS1 region (Zheng et al., 2010). The CNS1 deletion results in the reduction of iTreg cells, but not thymic Treg cells, indicating that the CNS1 region, which acts as a TGF-β sensor through binding of Smad3, is dispensable for thymic, but not peripheral, Foxp3 induction.

Thus, Treg-cell-specific epigenetic changes in thymic Treg cells are primed through the thymic T cell selection process, whereas the epigenetic alterations in in-vivo-produced iTreg

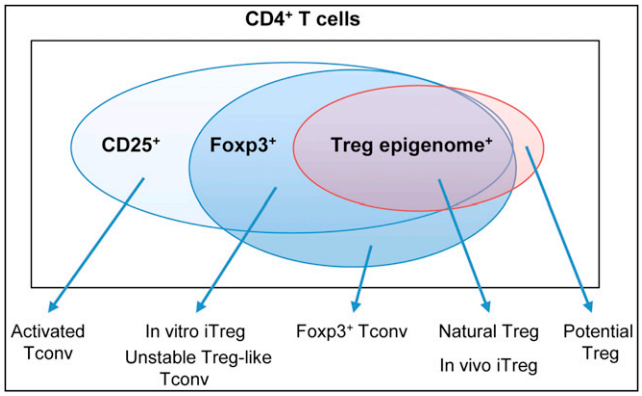

Figure 4. T Cell Subpopulations Delineated by CD25, Foxp3, and the Treg-Cell-Type Epigenome

The majority of Foxp3$^+$ T cells are CD25$^+$, and some are CD25$^-$. Foxp3$^+$ cells also contain T cells with or without Treg-cell-type epigenetic changes. Some T cells with the epigenetic changes are not Foxp3$^-$. Thymus-derived nTreg cells, in-vivo- or in-vitro-produced iTreg cells, and activated or naïve Tconv cells can be characterized by these molecular markers and events.

cells chiefly depend on extracellular stimuli, such as cytokines and other unknown stimulations. TGF-β-induced in vitro iTreg cells do not exhibit such changes.

Treg Cell Identity

The presence of Treg-cell-specific epigenetic changes prompts one to ask about their relationship with Foxp3 expression—more specifically, whether T cells possessing the Treg-cell-specific DNA hypomethylation pattern are identical to those expressing Foxp3 (Figure 4). Analysis with cell-fate-mapping reporter mice has demonstrated that a small T cell population that once expressed Foxp3 and then lost the expression but held DNA demethylation of the *Foxp3* CNS2 region was easily converted to functional Foxp3$^+$ Treg cells upon TCR stimulation (Miyao et al., 2012). In contrast, T cells possessing Foxp3 expression but not the Treg-cell-type DNA hypomethylation pattern fail to exhibit sufficient Treg cell suppressive activity and instead secrete effector cytokines upon loss of Foxp3 expression (Miyao et al., 2012; Miyara et al., 2009; Ohkura et al., 2012). In accordance with these findings, *Ets-1*-deficient mice, whose Foxp3$^+$ T cells lack Treg-cell-specific DNA demethylation of the *Foxp3* CNS2 region, show deficient Treg-cell-mediated immune suppression and develop autoimmunity (Mouly et al., 2010). Thus, together with Foxp3 expression, the Treg-cell-specific DNA hypomethylation pattern can be a reliable marker for defining functional Treg cells as a distinct cellular entity.

Interaction between the Transcriptional Network and the Epigenome

Genome-wide gene expression profiling in Treg cells has depicted a variety of transcription factors expressed in Treg cells and has argued that a certain combination of Foxp3 and other transcription factors, rather than Foxp3 expression alone, would be essential for determining Treg cell development and function (Hill et al., 2007; Samstein et al., 2012). Recently, Fu et al. (2012) have indicated that any combination of "quintet" transcription factors (e.g., IRF4, Eos, Lef1, Gata1, and Satb1) with Foxp3 can reproduce the Treg-cell-like gene expression profiles in Tconv cells. It has also been reported that genes directly regulated by Foxp3 are relatively limited (Hill et al., 2007), even though Foxp3-binding regions are frequently observed in a variety of loci, especially in promoter regions (Samstein et al., 2012; Zheng et al., 2007). Furthermore, Foxp3 has been identified as one of the constituents of large transcriptional complexes, which contain HDAC, Runx1, and Gata3 (Rudra et al., 2012). These observations suggest that genome-wide gene expression profiles of Treg cells are formed by combinations of transcription factors specifically assembled to compose transcription factor complexes in Treg cells. The idea might not be inconsistent with the concept that Treg cell development requires Treg-cell-specific epigenetic changes but rather complementary in the establishment of Treg-cell-specific gene expression profiles. Given the presence of common genes among the genes required for Treg-cell-type gene expression (Fu et al., 2012), those with Treg-cell-specific DNaseI-hypersensitive regions (Samstein et al., 2012), and those with Treg-cell-specific DNA-hypomethylated regions (Ohkura et al., 2012), it is conceivable that Treg-cell-type epigenetic changes influence the expression levels and stability of a small number of genes; then, those gene products might control the whole-gene expression pattern in Treg cells. It is possible that TCR stimulation, along with other extracellular stimulations such as cytokines and metabolic factors, might activate distinct signaling pathways and thereby determine the composition of the network of core transcription factors.

Treg Cell Plasticity and Adaptability

The plasticity or stability of Treg cells is controversial—in particular, regarding whether Foxp3-expressing nTreg cells, which are apparently more self-reactive than Tconv cells in their TCR repertoire, might differentiate into hazardous autoimmune effector T cells (Bailey-Bucktrout and Bluestone, 2011; Hori, 2011). For example, some Treg cells lose Foxp3 expression and differentiate into effector T cells (Komatsu et al., 2009; Xu et al., 2007; Yang et al., 2008; Zhou et al., 2009b). In addition, exposure to Th1- or Th17-cell-polarizing stimuli for a prolonged period modulates Treg cells to produce inflammatory cytokines and to lose suppressive activity (Xu et al., 2007; Zhao et al., 2011). However, findings with cell-fate-mapping reporter mice argue that "the plasticity" of Treg cells could be attributed to the presence of a minor population of Foxp3$^+$ T cells that lack *Foxp3* CNS2 demethylation (Miyao et al., 2012). Functional and phenotypic heterogeneity of FOXP3$^+$ cells is more evident in humans in that they include FOXP3^{hi} cells with potent suppressive activity and FOXP3^{lo} nonsuppressive cells capable of secreting proinflammatory cytokines (Miyara et al., 2009). Suppressive activities of these populations are well correlated with their DNA methylation status of the *FOXP3* CNS2 region.

Although Treg-cell-specific epigenetic changes are critical for Treg cell stability, extrinsic signals are also important for controlling Foxp3 expression and thus for maintaining Treg cells. For example, hypoxia-inducible factor 1 (HIF-1), a key metabolic sensor, regulates the balance between Treg and Th17 cell differentiation (Dang et al., 2011). HIF-1 is selectively expressed in Th17 cells, and its induction requires signaling through mTOR,

a central regulator of cellular metabolism (Shi et al., 2011). HIF-1 attenuates Treg cell development through an active process that targets Foxp3 for degradation. These findings indicate that metabolic-signal-dependent transcriptional regulation would be important for the lineage choices between Th17 and Treg cells. Complement fragments also affect the balance between Th17 and Treg cells. Signaling through the G-protein-coupled receptors for the complement fragments C3a and C5a in dendritic cells and $CD4^+$ cells enhances Th17 cell induction (Strainic et al., 2013). Conversely, when signals from C3aR and C5aR are inhibited in $CD4^+$ T cells, signal reduction in the PI3K-Akt-mTOR pathway and an increase in autocrine TGF-β signaling enhance $Foxp3^+$ iTreg cell generation. Moreover, Foxo transcription factors, which integrate extrinsic signals to regulate cell division, differentiation, and survival, have a pivotal role in the development of both thymic and induced Treg cells (Kerdiles et al., 2009; Kerdiles et al., 2010; Ouyang et al., 2010; Ouyang et al., 2012). Thus, extracellular stimulation would also be important for the stability and plasticity of $Foxp3^+$ Treg cells. It is, however, largely obscure whether these extrinsic stimulations can alter the Treg-cell-specific epigenetic pattern, given that Foxp3 expression and the establishment of the Treg-cell-type epigenetic pattern are independent events in the course of Treg cell development (Ohkura et al., 2012).

Recent work has demonstrated that Treg cells are able to adapt to environmental signals and further differentiate by expressing transcription factors normally associated with Th cell subsets (Campbell and Koch, 2011). The development of $T\text{-}bet^+$ Treg cells occurs in response to IFN-γ produced by effector T cells. T-bet induces the Th1-cell-associated chemokine receptor CXCR3, which enables the $T\text{-}bet^+$ Treg cells to migrate to a type 1 inflammation site and suppress local Th1 immune responses (Hall et al., 2012; Koch et al., 2009). T-bet-deficient Treg cells are indeed unable to accumulate at the site of Th1 inflammation and properly control the Th1 immune response. Additionally, $IRF4^+$ Treg cells control type 2 inflammation. Treg-cell-specific deletion of *Irf4* prevents Treg cells from efficiently suppressing Th2 immune responses, leading to uncontrolled Th2 immune responses (Zheng et al., 2009). Thus, it is likely that Treg and Tconv cells can respond to common differentiation stimuli and acquire some common phenotypes and function (e.g., migratory capacity to the same inflammation site). However, there is also a difference between the two populations in transcriptional regulation. For example, in Tconv cells, STAT1 activation by IFN-γ induces the expression of T-bet, which in turn drives expression of the IL-12 receptor component IL-12Rβ2 (Afkarian et al., 2002; Mullen et al., 2001). This allows the cells to undergo IL-12-dependent activation of *Ifng* and other genes required for proper function of Th1 cells. Koch et al. (Koch et al., 2009) revealed that in Treg cells, delayed induction of IL-12Rβ2 after STAT1 activation helps ensure that Treg cells do not readily complete Th1 cell differentiation and lose their suppressive ability. In that study, deletion of *Foxp3* in Treg cells failed to upregulate IL12Rβ2 expression, indicating that the repression of IL-12Rβ2 expression is mediated by a Foxp3-independent mechanism in Treg cells. Because Treg-cell-specific epigenetic changes contribute to Treg-cell-specific gene expression and occur in a small number of loci (Ohkura et al., 2012), the changes might allow Tconv-like behavior in Treg cells while keeping the expression of some core factors critical for Treg cell function.

As discussed above, the molecular and cellular basis of Treg cell stability and plasticity, as well as heterogeneity, remains a key issue of Treg cell research. For clinical use of functionally stable Treg cells for immunosuppression, it is necessary to know more precisely the developmental processes of Treg cells in terms of Foxp3 expression and epigenetic changes and determine the conditions under which these two events become discrepant and affect the functional stability of Treg cells. Furthermore, elucidation of the mechanisms underlying the induction of Treg-cell-specific epigenetic changes might provide new opportunities for pharmacological intervention.

Does Epigenetic Priming Occur in Other T Cell Subsets?

Similar to the case with Treg cells, epigenetics features of other T cell subsets have been investigated. After the differentiation of naïve $CD4^+$ T cells into helper T cells, new DNaseI-hypersensitive regions and specific DNA demethylation are observed at the *Ifng* or *Il4* locus in Th1 or Th2 cells, respectively, and de novo methylation takes place at the *Il4* or *Ifng* locus in Th1 or Th2 cells, respectively, to repress unwanted gene transcription (Ansel et al., 2003; Thomas et al., 2012). However, such clear-cut reciprocal epigenetic regulation is not seen between Th1 and Th17 cells; the distribution pattern of DNaseI-hypersensitive sites within the *Ifng* locus is similar between activated Th17 and Th1 cells, consistent with the ability of Th17 cells to produce IFN-γ under Th1-cell-polarizing conditions (Hirota et al., 2011; Mukasa et al., 2010). In addition, Th9, Th22, and Tfh cells were recently reported as distinct subsets of $CD4^+$ T cells given their unique expression of cytokines or transcription factors (Annunziato and Romagnani, 2009; Fazilleau et al., 2009). Thus, it remains to be determined how the epigenetic status of these various Th cell subpopulations contributes to their lineage stability and plasticity in cytokine production.

Conclusions and Perspectives

As discussed in this review, Foxp3 induction and the establishment of the Treg-cell-type epigenetic pattern are independent but complementary events in the course of Treg cell development. Furthermore, Treg-cell-specific epigenetic changes, especially DNA hypomethylation, make a cell poised for expressing not only Foxp3 but also other Treg-cell-function-associated molecules and contribute to the stability of cell lineages that are critical for long-term immune tolerance. Treg-cell-type epigenetic change is therefore a key molecular event for defining Treg cells as a functionally distinct T cell subpopulation. It is also vital for preparing functionally stable $Foxp3^+$ Treg cells for clinical use. It has been shown that Foxp3 can be induced in Tconv cells by a variety of molecules, such as TGF-β, retinoic acids, rapamycin, and sphingosine 1-phosphate receptor agonist FTY720 (Battaglia et al., 2006; Hill et al., 2008; Mucida et al., 2007; Zhou et al., 2009a), and that several signaling pathways, including those of NFAT, Smad3, and the Nr4a family, are critical for inducing Foxp3 expression (Sekiya et al., 2011; Sekiya et al., 2013; Tone et al., 2008; Xu et al., 2010). Regarding Treg-cell-type epigenetics, it remains to be determined how locus-specific DNA demethylation occurs in the course of Treg cell development and what types of extracellular stimulations can install Treg-cell-type

epigenetic changes. In addition, elucidating the crosstalk between different epigenetic modifications, including chromatin remodeling, histone modifications, and DNA methylation, and their respective roles in epigenetic regulation is essential for our understanding of the contribution of Treg-cell-type epigenetic changes to Treg cell development and function. This will enable better control of immune responses in clinical settings via targeting the generation and maintenance of thymus-derived and peripherally induced Treg cells.

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